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Multiple Forms of Arylalkylamine N-Acetyltransferases in the Rat Pineal Gland: Purification of One Molecular Form

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Rat pineal serotonin N-acetyltransferase (EC 2.3.1.87) activity is isolated in two molecular forms ($M_r \cong$ 10,000 and 95,000) by high performance size exclusion liquid chromatography in the presence of ammonium acetate (0.1 M, pH 6.5). In the presence of sodium citrate (0.1 M, pH 6.5), however, it is eluted as a single peak of intermediate size ($M_r \cong$ 30,000). A highly enriched preparation of one of the molecular forms has been obtained by a two-step purification procedure involving disulfide-exchange and anion-exchange chromatography. The N-acetyltransferase in 250 pineal glands obtained from isoproterenol-treated rats can be purified about 80-fold in 1 day; recovery is about 3%. Polyacrylamide gel electrophoresis of the final preparation indicates that a single major band ($M_r \cong$ 11,000) is present; this appears to be serotonin N-acetyltransferase.

Key words: cystamine, disulfide exchange, fast protein liquid chromatography, anion exchange, multiple forms

INTRODUCTION

Pineal serotonin N-acetyltransferase (arylalkylamine N-acetyltransferase, EC 2.3.1.87; NAT) is the first enzyme in the metabolic conversion of

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serotonin to melatonin, the putative pineal hormone [Weissbach et al., 1960]. It is of special interest that the activity of this enzyme increases many fold at night; in the rat this increase is 50- to 100-fold [Axelrod and Zatz, 1977; Klein, 1978]. A neural circuit involving both central and peripheral neural structures controls this increase [Klein et al., 1981]. Sympathetic nerves in the gland release norepinephrine, which is known to act through an adrenergic-cyclic AMP mechanism to increase NAT activity [Axelrod and Zatz, 1977; Klein, 1978]. This is thought to involve both induction, perhaps of NAT, and stabilization of this enzyme [Romero et al., 1975; Klein et al., 1981]. When neural stimulation of the pineal gland ceases, the enzyme is rapidly inactivated ($t_{1/2}$ = 3 to 4 min) [Klein and Weller, 1972]; indirect studies have suggested that protein thiol:disulfide exchange may be involved [Binkley et al., 1976; Namboodiri et al., 1980, 1981). These large changes in NAT activity control the daily rhythm in pineal melatonin production and melatonin in the circulation [Klein, 1978; Reppert and Klein, 1980].

Little progress has been made in determining the precise molecular mechanisms involved in NAT regulation, i.e., whether the enzyme is induced by adrenergic stimulation and if protein thiol:disulfide exchange is involved in the inactivation. The reason for this lack of understanding, in part, is that purified preparations of the enzyme have not been available. Purification has been difficult because of the highly unstable nature of the enzyme [Binkley et al., 1976] and because the tissue is small and difficult to obtain in large quantities. Recently, however, we succeeded in developing a simple and rapid procedure to purify ovine pineal NAT. This involves disulfide-exchange and anion-exchange chromatography steps [Namboodiri et al., 1987]. We used a modification of this approach in the present study and were able to isolate one molecular form of the enzyme.

MATERIALS AND METHODS

Pineal glands were obtained from male Sprague-Dawley rats (100 to 125 g; Zivic Miller Co., Allison Park, PA). Animals were injected twice with DL-isoproterenol (10 mg/kg), with a 2 hr interval between injections. Rats were killed 2 hr after the second injection. Pineal glands were rapidly removed and placed on solid CO_2 immediately.

Sepharose-cystamine was synthesized using cyanogen bromide-activated Sepharose [Namboodiri et al., 1987]. The high performance size exclusion liquid chromatography column (TSK 3000) was purchased from Kratos Corporation (New York, NY) and the anion-exchange (MonoQ) column from Pharmacia Fine Chemicals (Piscataway, NJ). Radiolabeled molecular weight markers and the Bolten-Hunter reagent were obtained from New England Nuclear Corporation (Boston, MA). The bovine serum albumin (BSA) was afraction V preparation (Sigma Chemical Co., St. Louis, MO). Other chemicals were obtained from commercial sources. All procedures were done at 0° to 4°C.

Homogenate Preparation

Homogenates of rat pineal glands were prepared by sonication (1 gland/10 μl) in 10 mM ammonium acetate, pH 6.5; glands were sonicated in groups

of 20. The homogenate was centrifuged (1 min, 10,000 g), and the supernatant was used as the starting material. This preparation contained about 95% of the enzyme activity originally present in the homogenate.

Enzyme Assay

NAT activity was assayed by incubating (37°C; 30 min) a 50 μl sample with 50 μl of 100 mM sodium phosphate buffer, pH 6.8, containing L-[^{14}C]acetyl CoA (0.24 mM; SA = 8.3 Ci/mol) and tryptamine (20 mM) [Deguchi and Axelrod, 1972; Parfitt et al., 1975]. The reaction was terminated by addition of 1 ml of chloroform, which extracts the product, N-[^{14}C]acetyltryptamine. The aqueous layer was removed, and the chloroform was washed once with 0.2 ml of 100 mM sodium phosphate buffer, pH 6.8, and twice with 0.2 ml of 1 N NaOH. A sample of chloroform was taken to dryness, and radioactivity was measured by routine methods.

Disulfide-Exchange Liquid Chromatography

A chromatographic column (0.5 × 5 cm) containing Sepharose-cystamine was equilibrated with 10 mM veronal-HCl buffer, pH 8.5, containing 100 mM sodium citrate. A 2.5 ml sample of the supernatant was mixed with 5 ml of 30 mM veronal-HCl buffer, pH 8.5, containing 300 mM sodium citrate, and then loaded (10 ml/hr). The effluent was reapplied to the column twice to maximize binding. The column was then washed (100 ml/hr) sequentially with 10 ml of the equilibrating buffer, 20 ml of 100 mM sodium citrate, pH 6.5, and finally with 20 ml of 25 mM ammonium acetate buffer, pH 6.5, containing 10 mM dithiothreitol (DTT). The flow was then stopped; 6 to 8 hr later washing was continued with the same buffer (about 50 ml) until the eluate was protein free. Enzyme activity was then eluted using either 20 mM Tris HCl, pH 8.5, containing 1 mM DTT or 20 mM ATP, pH 6.5, containing DTT (10 mM). The eluate was collected in 0.50 ml fractions; 90% of the eluted enzyme activity was usually found in the first eight fractions.

Anion-Exchange High Pressure Liquid Chromatography (HPLC)

A MonoQ column was equilibrated with Tris HCl (20 mM, pH 8.3) containing DTT (1 mM) and glycerol (10%) using a Gilson HPLC system. A 5 to 10 ml sample of the pooled fractions from the previous step was passed over the column (flow rate = 1 ml/min), the column was washed using the equilibrating buffer (10 ml), and enzyme activity was eluted using a linear gradient of NaCl (0 to 1.0 M; 30 min; 1 ml/min) in Tris HCl (20 mM, pH 8.4) containing DTT (1 mM) and glycerol (10%). Fractions (1 ml) were collected and assayed for NAT activity.

Size Exclusion HPLC

Two columns were used in series, and different buffers (100 mM sodium citrate, pH 6.5, and 100 mM ammonium acetate, pH 6.5, both containing BSA [0.1 mg/ml] and DTT [10 mM] were used as described in the text. The flow rate was 1 ml/min. The column was equilibrated with the buffer by passing 5 to 10 volumes through the system. A 1 ml sample of the enzyme

preparation was introduced, and 1 ml fractions were collected and assayed for NAT activity.

Concentration of Samples Using Ultrafiltration

Disposable ultrafiltration units (immersible CX-10; Millipore Corporation, Bedford, MA) were used. The units were first soaked in sodium citrate buffer, pH 6.5, containing BSA (0.1 mg/ml) for 10 min. Samples were prepared by mixing 9 volumes of the enzyme preparation with 1 volume of 1 M sodium citrate, pH 6.5, containing BSA (1 mg/ml). In a typical run using the adenosine triphosphate (ATP) eluate, the volume was reduced from 5 to 1 ml in 30–40 min, with nearly complete recovery of enzyme activity.

Trichloroacetic Acid Precipitation of Proteins

A 250 μl sample of trichloroacetic acid (TCA) (50%) was added to a 1 ml sample containing sodium deoxycholate (0.1%). After 1 hr (0–2 °C), the sample was centrifuged (10,000g, 10 min) and washed twice with 1 ml volumes of acetone.

Radioiodination of Proteins

Proteins were labeled using the [^{125}I]Bolten-Hunter reagent as previously described [Bolten and Hunter, 1973] with the following minor modifications. The TCA-precipitated protein sample was dissolved in 25 μl of 0.1 M borate buffer, pH 8.5, added to a "Combi-U-vial" containing the air-dried iodinated ester, agitated, and kept at 0°C for 18 hr to complete the reaction. Prior to electrophoresis the proteins were reprecipitated using TCA as described above.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

Proteins in samples of crude or purified rat NAT were precipitated with TCA and solubilized (90°C, 5 min) in 40 μl of sample buffer (2.5% SDS, 5% β-mercaptoethanol, 10% glycerol, 60 mM Tris HCl, pH 6.8). The samples were applied to a 15% acrylamide, 0.1% SDS slab gel with a 3% acrylamide stacking gel, and electrophoresis was run at 20 mA constant current [Laemmli, 1970]. The gels were fixed in 50% methanol containing 10% acetic acid overnight. Silver staining was performed by a modification of a published procedure [Morrissey, 1981] with successive incubations in 5% glutaraldehyde (30 min), 0.1 mM DTT (30 min), 0.2% silver nitrate (30 min), and 3% sodium carbonate containing 0.02% formaldehyde (3–10 min). Commassie brilliant blue-R staining was performed using a standard procedure [Sarkar and Dion, 1975] in 45% methanol containing 10% acetic acid. When autoradiography was required, the gels were dried and exposed to a Kodak XAR film between two intensifying screens for 2–24 hr.

Protein Estimation

Proteins were estimated using a microdye-binding procedure in 96 well microtiter plates with BSA as the standard [Bradford, 1976]. The color was read using an automatic Elisa reader (Flow Labs, McLean, VA).

RESULTS

Multiple Forms of Rat Pineal NAT Resolved by Size Exclusion Chromatography

A 1 ml aliquot of the ATP eluate from the Sepharose-cystamine column was passed over the TSK column equilibrated with ammonium acetate (0.1 M, pH 6.5) containing DTT (10 mM) and BSA (0.1 mg/ml). Enzyme activity was detected in two peaks ($M_r \cong 10{,}000$ and $\cong 95{,}000$), with about 70% recovery of activity (Fig. 1A). In contrast, when a similar ATP eluate of the enzyme was passed over the TSK column equilibrated with sodium citrate (0.1 M, pH 6.5) containing DTT (10 mM) and BSA (0.1 mg/ml), the enzyme activity was detected in one peak ($M_r \cong 30{,}000$); recovery of activity was about 80% (Fig. 1B). The fractions containing enzyme activity were pooled, concentrated, and passed over the ammonium acetate-equilibrated TSK column under the same conditions as above. Enzyme activity was detected in two peaks ($M_r \cong 10{,}000$ and $\cong 95{,}000$), most of the activity being detected in the low molecular weight form with a recovery of activity of about 50% (Fig. 1C). In several experiments we have consistently observed that pretreatment of the ATP eluate with sodium citrate (0.1 M, pH 6.5) reduces the proportion of the high molecular weight form obtained on the ammonium acetate-equilibrated TSK column chromatography, apparently as a result of conversion of the high molecular weight form to the small form.

Purification

The procedure for the purification of rat pineal NAT involves two chromatographic steps (Table 1). The first, disulfide-exchange chromatography, provides about 17-fold purification with about 65% recovery of activity. The second step, anion-exchange chromatography using a MonoQ column, is very rapid and yields about 50% recovery of activity in three overlapping peaks, one of which is relatively uncontaminated with other proteins. The entire procedure requires 1 day. Overall recovery of activity is about 30% and that calculated for the purest form of the enyzme is about 3%. A detailed description of the procedure follows.

Disulfide-exchange chromatography using Sepharose-cystamine. A summary of the disulfide-exchange chromatography is given in Table 2. Near complete binding of enzyme activity on the column occurs under the conditions of loading. No significant amount of the enzyme, in either the active or inactive form as detected by its ability to be reactivated by DTT treatment, is found in the effluent. A small quantity of the enzyme and proteins are released after reduction of the mixed disulfide by DTT. Washing the column using Tris HCl, pH 8.4, containing DTT (1 mM) elutes about 65% of the enzyme activity loaded onto the column.

Anion-exchange chromatography. The chromatographic profile of the anion-exchange procedure indicated that almost all the proteins, including the enzyme, bind to the column under the conditions of loading; no enzyme activity or protein is detected either in the effluent or the wash. Three peaks of NAT activity are detected in the eluate (Fig. 2). The first (peak I) appears at the start of the gradient (30–90 mM NaCl). Most protein is eluted following peak I.

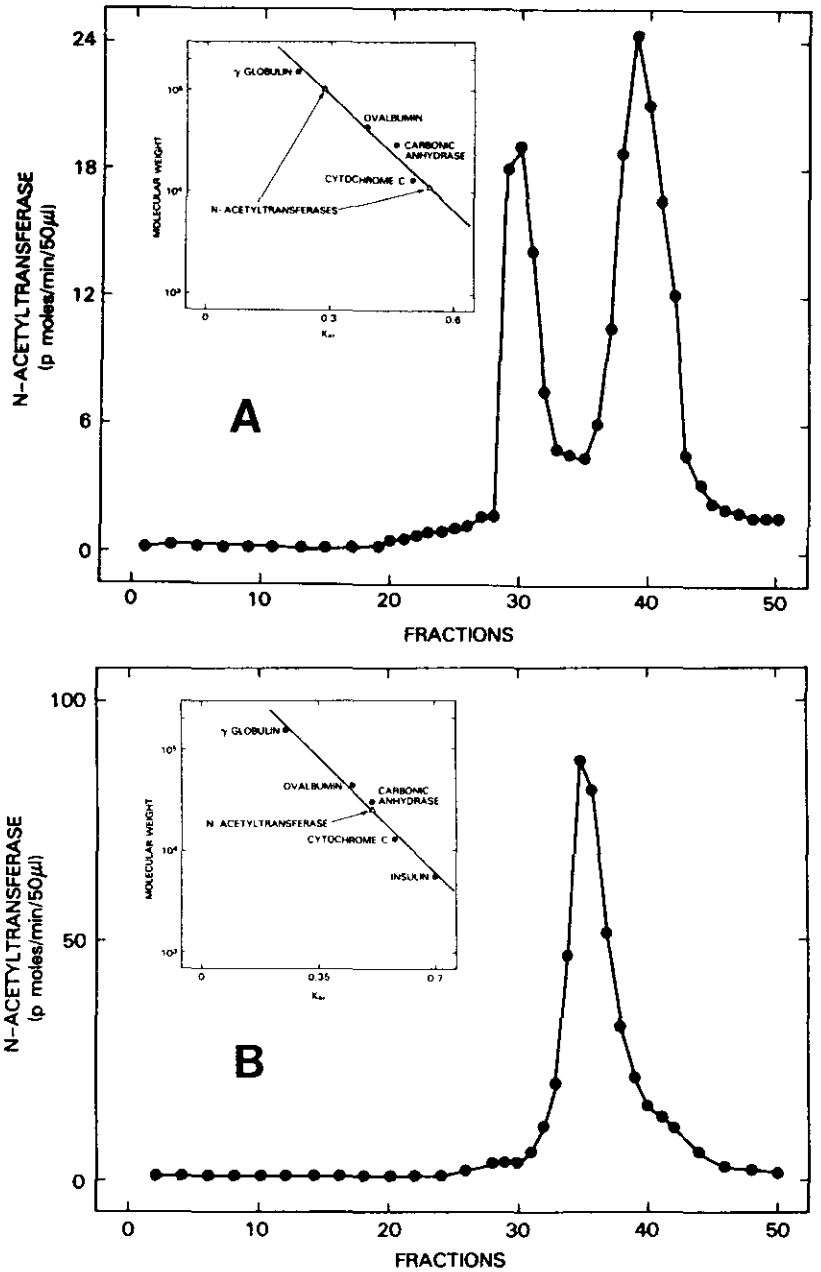

Fig. 1. Size exclusion chromatography of rat pineal NAT. Chromatography was done on TSK (3000) columns, as described, in the presence of 0.1 M ammonium acetate, pH 6.5 (**A**,**C**) and 0.1 M sodium citrate, pH 6.5 (**B**). A 1 ml aliquot of ATP eluate from a Sepharose-cystamine column was used for the procedures in A and B. Five fractions (1 ml each) containing the enzyme activity from the procedure in B were pooled, concentrated to 1 ml, and the sample was loaded on to the TSK column equilibrated with 0.1 M ammonium acetate, pH 6.5 (**C**). Recoveries of activity in the three procedures were A, 70%; B, 80%; and C, 50%. To determine the molecular weight of the enzyme, a 1 ml sample containing the molecular standards was passed through the column under the same conditions used for the three procedures described above; the positions of the standards were determined. Molecular sizes of the peaks of NAT activity were determined graphically, as indicated.

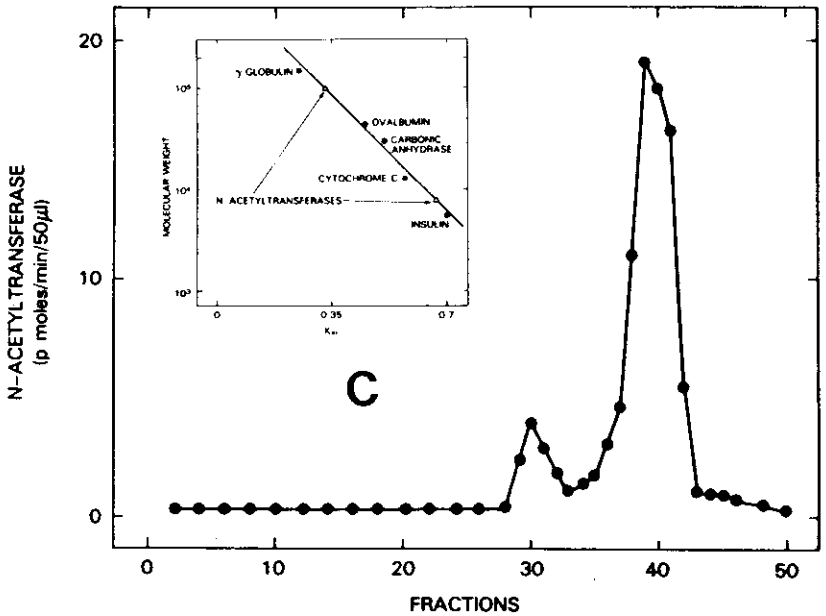

Figure 1. Continued

TABLE 1. Summary of the Purification of Rat Pineal NAT

Purification step	Enzyme activity (nmol/min)	Protein (mg)	Specific activity (nmol/min/mg protein)	Recovery (%)	Fold purification
Crude	31.4	28.5	1.1	100	—
Sepharose-cystamine	20.7	1.1	18.8	66	17
MonoQ	0.83	0.010	83	3	76

Purification was done as described in Materials and Methods, using 250 pineal glands from isoproternol-treated rats. Enzyme activity was measured at each step using 50 μl samples and protein using 10 to 100 μl samples. Similar results were obtained three times.

Analysis. The protein patterns revealed by SDS-PAGE of samples obtained at sequential steps of a typical purification are presented (Fig. 3). Using Commassie blue stain, the fractions obtained from Sepharose-cystamine columns showed enrichment of proteins at $M_r \cong 45{,}000$, $\cong 26{,}000$, and $\cong 11{,}000$ (Fig. 3A). A single band ($M_r \cong 11{,}000$) was detected in peak I when stained using Commassie blue, although attempts to stain this band with silver were often unsuccessful; in a single experiment silver staining, after a dehydration-rehydration cycle of the gel, revealed a band of protein at $M_r \cong 11{,}000$. Peak I protein was also labeled with [^{125}I]Bolten-Hunter reagent and analyzed by SDS-PAGE. Visual inspection of the autoradiogram revealed one to five bands according to the exposure time. An intensely labeled band at $M_r \cong 11{,}000$ was obtained in all cases. Scanning with a densitometer revealed that this band represents about 75% of the proteins resolved on the gel (Fig. 3B).

TABLE 2. Purification of Rat Pineal NAT Using Sepharose-Cystamine

Chromatographic fraction	Enzyme activity (nmol/min)	% Load	Protein (mg)	% Load	Specific activity (nmol/min/mg protein)	Fold purification
Crude	31.4	100	28.5	100	1.1	—
Effluent	0.9	3	20.5	72	—	—
DTT wash	2.7	9	1.7	6	1.6	1.5
pH eluate	20.7	66	1.1	4	18.8	17

NAT activity and proteins were measured in 50 μl aliquots. Values of activity and proteins for the fractions from the loading step and the first wash step using the equilibrating buffer are given under "Effluent." No proteins could be detected in the sodium citrate, pH 6.5, wash and the first ammonium acetate-DTT wash; the values for the second ammonium acetate-DTT wash are given under "DTT wash." For further details see Materials and Methods.

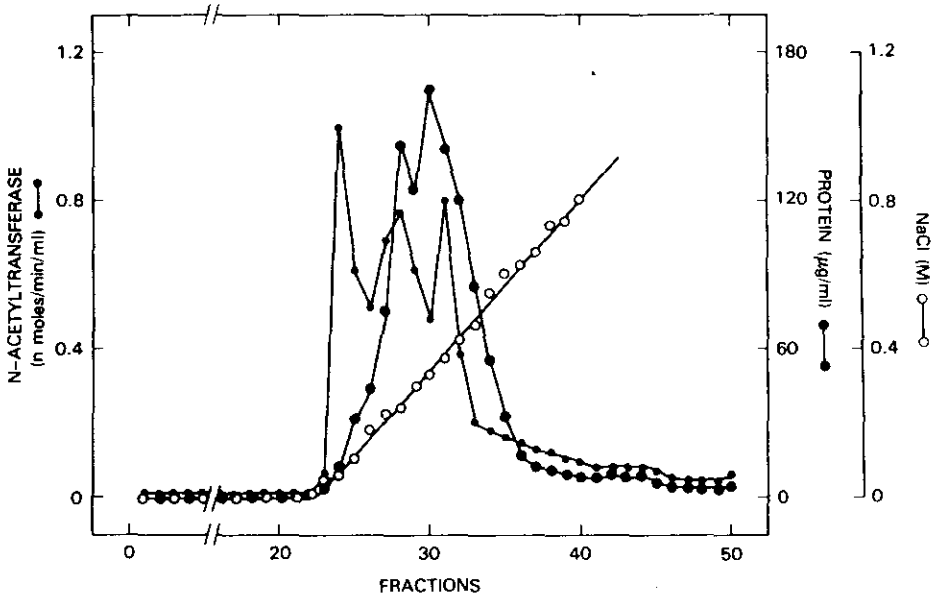

Fig. 2. Purification of rat pineal serotonin NAT by anion-exchange chromatography using MonoQ columns. Purification was done as described in Materials and Methods. Enzyme activity was measured in aliquots of 100 μl and protein in 50 μl. Concentration of NaCl was estimated by measuring conductivity, with a Markson Portable Conductivity Meter.

DISCUSSION

In the present report chromatographic evidence is provided that shows that rat pineal NAT can exist in three molecular forms ($M_r \cong$ 10,000, 30,000, and 95,000) depending on the ionic environment. In addition, we have presented a simple and rapid procedure to purify one of these forms. Two topics will be discussed here. The first relates to the new information about rat pineal NAT revealed by the present study and the second to the purification of one of the three molecular forms.

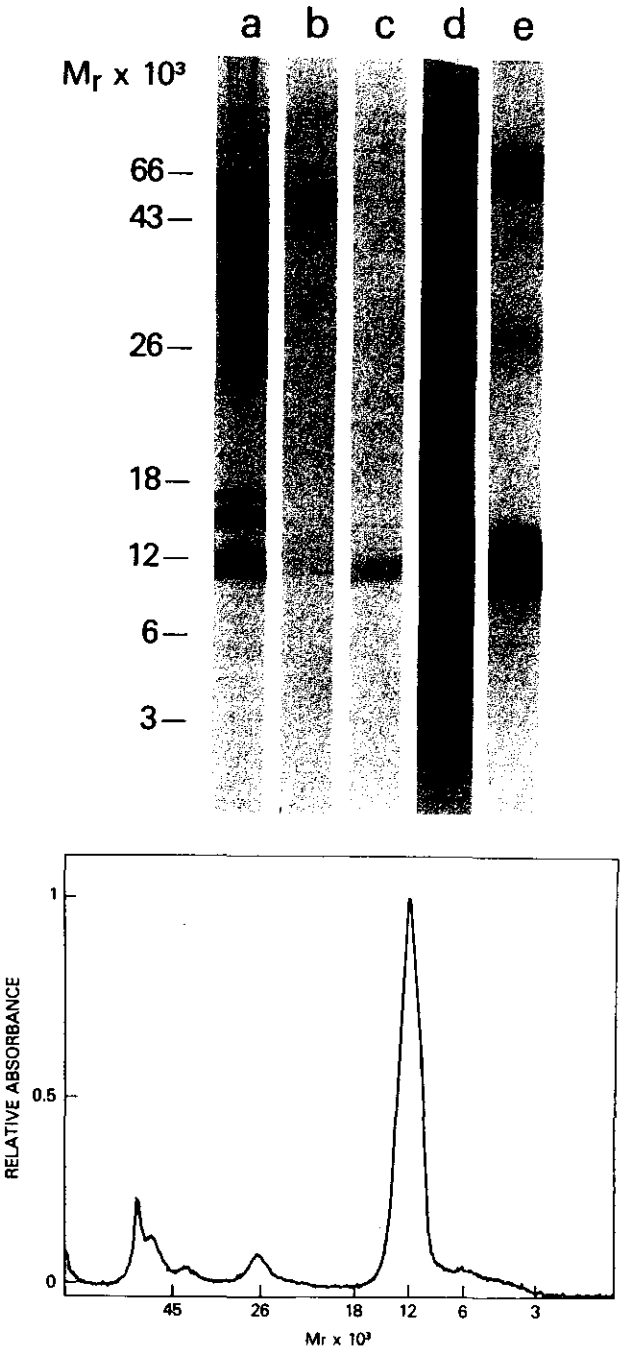

Fig. 3. SDS-PAGE patterns of samples at sequential stages of purification. Samples were analyzed on a 15% acrylamide slab gel. **Top:** 1) 10,000g supernatant, 100 μg of protein, Commassie blue-R stain. b) Pooled fractions containing NAT activity from cystamine column, 15 μg of protein, Commassie blue-R stain. c) Anion-exchange fraction of highest purity, 6 μg of protein, Commassie blue-R stain. d) Anion-exchange fraction of highest purity, 3 μg of protein, silver stain. e) Anion-exchange fraction of highest purity, 3 μg of protein autoradiography after [^{125}I]Bolton-Hunter reagent labeling. **Bottom:** The autoradiogram from Lane e was scanned with a soft laser scanning densitometer. The protein band observed at 11,000 daltons accounted for 75% of the labeled material present on the gel. Molecular mass standards used were ovalbumin, 43,000 daltons; α-chymotrypsin, 25,700 daltons; β-lactoglobulin, 18,400 daltons; cytochrome *c*, 12,000 daltons; bovine trypsin inhibitor, 6,200 daltons; insulin α and β chains, 3,000 daltons.

Size exclusion chromatography yielded important information on the possible molecular forms of NAT and their interconversions. On the basis of the results of size exclusion chromatography it appears that like the sheep enzyme [Namboodiri and Klein, 1981], the rat enzyme can exist in three molecular forms, depending on the ionic environment. This is supported by our observation that the enzyme can be separated into three peaks of activity on MonoQ columns. We also have been able to detect the three forms of the enzyme in crude preparations [Voisin et al., 1984]. However, in the presence of ammonium acetate (0.1 M, pH 6.5), the proportion of the higher molecular weight form ($M_r \cong 95{,}000$) was consistently observed to be higher than that of the small form ($M_r \cong 10{,}000$), unlike the pattern observed after the Sepharose-cystamine step. In an earlier report [Morrissey et al., 1977] it was shown that rat pineal NAT in 105,000g supernatant fraction can be separated into two forms ($M_r \cong 39{,}000$ and 10,000) on Sephadex G100 chromatography in the presence of potassium phosphate (50 mM, pH 6.5) containing β-mercaptoethylamine (4 mM). Our results show that the multiple forms of the enzyme depend on the ionic environment, whereas the results of Morrissey et al. [1977] seem to indicate that interaction between sulfhydryl groups is involved in this process. Although further investigations are required to resolve this controversy, both sets of results indicate that molecules of NAT may exist within the pineal cells in polymeric forms.

It should be emphasized that our results show that the important variable in the ionic environment influencing the chromatographic behavior of NAT is the nature of the ion, not ionic strength. In a previous report on the purification of sheep pineal NAT it was found that increasing the concentration of ammonium acetate from 50 to 400 mM did not convert the $M_r \cong 10{,}000$ and $M_r \cong 95{,}000$ forms of the enzyme to the $M_r \cong 30{,}000$ form, whereas treatment with 100 mM citrate did [Namboodiri and Klein, 1981]. It is interesting that these effects of salts appear not to be related to their effects on activity: We have found that increasing the ionic strength of buffers by adding sodium chloride, sodium citrate, sodium phosphate, ammonium acetate, postassium chloride, or ATP increases enzyme activity as much as fivefold [Namboodiri et al., 1979]. Thus, it appears there are two apparently independent effects of ions on NAT. All salts appear to activate the enzyme in a dose-dependent manner, perhaps through a common mechanism involving hydration or charge density of the active site. In contrast, it appears that certain salts (e.g., sodium citrate) that stabilize the enzyme [Namboodiri et al., 1979] can shift the enzyme from the $M_r \cong 10{,}000$ and $M_r \cong 95{,}000$ forms to the $M_r \cong 30{,}000$ form; other salts, including ammonium acetate, can convert the $M_r \cong 30{,}000$ form to the other two forms. One obvious difference between these salts is that one is monoanionic and the other is polyanionic. Perhaps this is the critical difference that determines the form of NAT in solutions of these salts. It is also possible that the apparent sensitivity of NAT to differences in the salt composition may play an important role in the intracellular form, activity, and stability of this enzyme.

On the basis of the molecular weight data presented in this report, we believe that the $M_r \cong 11{,}000$ band on SDS-PAGE is NAT and that the other molecular forms detected on size exclusion chromatography represent its

polymers or complexes with other proteins. The $M_r \cong 11{,}000$ subunit might form an $M_r \cong 33{,}000$ trimer and an $M_r \cong 99{,}000$ nonomer.

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